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Nucleotide Receptors in the Nervous System

An Abundant Component Using Diverse Transduction Mechanisms

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Abstract

Extracellular nucleotides achieve their role as cell-to-cell communicators by acting at cell surface transmembrane receptors—the P2 receptors. Before molecular cloning led to the isolation of any P2-receptor sequence, a small number of receptor types had been proposed on the basis of pharmacological evidence. The application of molecular biology to this field of receptor research has indicated that a great underestimation of the number of receptor subtypes and of their abundance had occurred. There are now known to be seven characterized P2Y (G protein linked) receptors and the same number again of P2X receptors of the transmitter-gated ion channel type. In this review, we discuss the properties of these cloned receptors, their distribution within the nervous system, and their methods of signal transduction.

Index Entries: ATP; P2Y receptor; P2X receptor; nucleotides; G protein-linked receptor; transmitter-gated ion channel; localization; functional characterization.

Introduction

Specific receptors for extracellular ATP that recognize the triphosphate nucleotide but not adenosine were first proposed by Burnstock (1972), on the basis of pharmacological evidence from autonomic neuroeffector cells. Such evidence, and the range of cell types involved, were subsequently much extended (reviewed in the volume edited by Dubyak and Fedan, 1990). The status of ATP as a neurotransmitter was supported by evidence for its release at the responsive sites, either in a paracrine mode, as in the vascular system, or from both peripheral and central nerve terminals (reviewed by Dubyak and El-Moatassim, 1993; Harden et al., 1995). The postulated receptors were termed P2 purinoceptors, to distinguish them from the known adenosine receptors (termed P1 purinoceptors). At an early stage it was recognized from physiological studies that there was more than one category of ATP receptor, and two subtypes, P2Y and P2X were named by Burnstock and

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Kennedy (1985). The definitions of these rested originally on agonist selectivities: in particular 2-methylthio-ATP (2MeS-ATP) was much more potent than αβ-methylene ATP (αβ-meATP) for P2Y and vice versa for P2X receptors. Various smooth muscle types were considered to exhibit prototypical P2Y or P2X responses. In fact, we can now see that it was a most fortunate historical accident that the pharmacological division was created thus, since it happens to coincide with a fundamental division in molecular structure.

The derivation since 1993 of DNA clones coding for nucleotide receptors has (as detailed in this review) transformed our view of these receptors and their subtypes. A range of genes, of the order of 14 in total so far known, has been shown to produce these receptors. The receptor originally identified as P2Y for its preference for 2MeS-ATP and being unaffected by αβ-meATP, actually comprises multiple subtypes, which belong to the larger family of G protein-linked (or metabotropic) nucleotide receptors. The other subclass of this family (P2X), originally recognized by αβ-meATP responsiveness (e.g., those in the rat vas deferens and those mediating vasoconstriction in many vascular smooth muscles) are now known to belong to a larger family of ionotropic ATP receptors. Those two families (P2Y and P2X) have nothing in common except a binding site for ATP, which itself is created by entirely different structures in each family. The particular agonists which had been used for distinguishing P2Y and P2X receptors turn out, in reality, to be far from diagnostic: 2MeS-ATP (or its breakdown product 2MeS-ADP) ranges in activity among metabotropic P2Y receptors, being very effective ($P2Y_1$) to virtually inactive ($P2Y_2$), whereas on most of the ionotropic P2X receptor subtypes now known, αβ-meATP has little or no activity.

The P2Y Receptors

The P2Y receptors constitute a new subfamily within the rhodopsin type superfamily of the G protein-linked receptors and contain all of the typical features of such receptors, including of course the seven hydrophobic transmembrane (TM) domains (TM1–TM7). To date, seven sequences, each from a different gene, have been isolated that encode polypeptides of 308–532 amino acids in length that recognize nucleotides (Table 1, Figs. 1A, 2). Within the P2Y family, the members are highly diverse in sequence, sharing 27–60% amino acid sequence identity. The coding regions of all receptors are intronless where this has been investigated.

Four subfamilies can be distinguished so far on the basis of their sequence similarities and in some cases their pharmacological properties (Figs. 1, 5). Three of these—$P2Y_1$, $P2Y_2$ / $P2Y_4$ / X1-P2Y, and $P2Y_3$ / $P2Y_6$—have a considerable number of residues conserved in all of them, and this is regardless of species (Fig. 2 and mapped in Fig. 4 of Barnard et al., 1996, where R5 is $P2Y_4$). In contrast, $p2y_5$ differs from all of these three at some of those residues. $p2y_5$ remains a provisional family member at present, having been little investigated, but $P2Y_1$ through $P2Y_4$ and $P2Y_6$ and X1-P2Y have been shown to be able to couple through the inositol phosphate (IP_3) pathway. The third intracellular loop and the C-terminus tail, regions implicated in G protein specificity in other G protein-linked receptors, vary greatly between all of those five sequences.

Properties of the P2Y Receptors

The $P2Y_1$ Receptor

A cDNA encoding a protein of 362 residues was originally cloned from late-embryonic chick brain and confirmed to be a P2Y-type receptor, by oocyte expression (Webb et al., 1993). In $P2Y_1$-transfected COS-7 cells, agonist application leads to the formation of IP_3 (Simon et al., 1995a), which is the common signal transduction pathway for this receptor type. ATP, ADP, and many of their substituted derivatives were found to agonists at this receptor, with 2MeS-ATP the most potent ago-

Table 1
Database Listings of P2Y and P2X Receptor Sequences

Receptor	Species	Accession number	References
$P2Y_1$	Chicken	X73268	Webb et al., 1993
	Turkey	U09842	Filtz et al., 1994
	Bovine	X87628	Henderson et al., 1995
	Mouse	U22829	Tokuyama et al., 1995
	Rat	U22830	Tokuyama et al., 1995
	Human	U42029	Ayyanathan et al., 1996b[a] (short form)[c]
	Human	U42030	Ayyanathan et al., 1996b[a] (long form)[c]
	Human	Z49205	Leon et al., 1996[a]
	Human	Jc4737	Janssens et al., 1996[a]
$P2Y_2$	Mouse	L14751	Lustig et al., 1993
	Mouse	S83099	Enomoto et al., 1996
	Human	U07225	Parr et al., 1994
	Rat	U09402	Rice et al., 1995
	Rat	L46865	Chen et al., 1996
	Rat	U65839	Seye et al., unpublished[a]
$P2Y_3$	Chicken	C98283	Webb et al., 1996b
$P2Y_4$	Human	X91852	Communi et al., 1995
	Human	U40223	Nguyen et al., 1995
	Human	X96597	Stam et al., 1996a
$p2y_5$	Chicken	I50241	Webb et al., 1996c[a,b]
	Human	AF000546	Bohm et al., unpublished[a]
$P2Y_6$	Rat	Q63371	Chang et al., 1995
	Human	U52464	Southey et al., 1996[a]
	Human	X97058	Communi et al., 1996b
X1-P2Y	Xenopus	X99953	Bogdanov et al., 1997
$p2y_9$ or	Human	U90322;	Bohm et al., unpublished[a]
$p2y_5$-like	Human	AF005419	Janssens et al., 1997
$p2y_{10}$	Human	AF000545	Bohm et al., unpublished[a]
$P2X_1$	Rat	X80477	Valera et al., 1994
	Mouse	X84896	Valera et al., 1995
	Human	X83688	Valera et al., 1995
	Human	U45448	Longhurst et al., 1996
$P2X_{2(a)}$	Rat	U14414	Brake et al., 1994
$P2X_{2(b)}$	Rat	Y09910	Brändle et al., 1997
	Rat	Y10473	Simon et al., 1997a
$P2X_{2(c)}$	Rat	Y10474	Simon et al., 1997a
$P2X_{2(d)}$	Rat	Y10475	Simon et al., 1997a
$P2X_3$	Rat	X90651	Chen et al., 1995
	Rat	X91167	Lewis et al., 1995
$P2X_4$	Rat	X87763	Buell et al., 1996
	Rat	X91200	Bo et al., 1995
	Rat	X93565	Soto et al., 1996a
	Rat	U32497	Seguela et al., 1996
	Rat	U47031	Wang et al., 1996
	Human	Y07684	Garcia-Guzman et al., 1997
	Human	AF000234	Takahashi et al., unpublished
$P2X_5$	Rat	X92069	Collo et al., 1996
	Rat	X97328	Garcia-Guzman et al., 1996
$p2x_{5(a)}$	Human	U49495	Unpublished[a]
$p2x_{5(b)}$	Human	U49396	Unpublished[a]
$P2X_6$	Rat	X92070	Collo et al., 1995
	Rat	C97376	Soto et al., 1996
$P2X_7$	Rat	X95882	Suprenant et al., 1996
	Human	Y09561	Rassendren et al., 1997

[a]No functional data, assignment based on sequence homology and [b]binding data.
[c]This receptor sequence has a short 3' UTR.
[d]Alternate long 3' UTR.

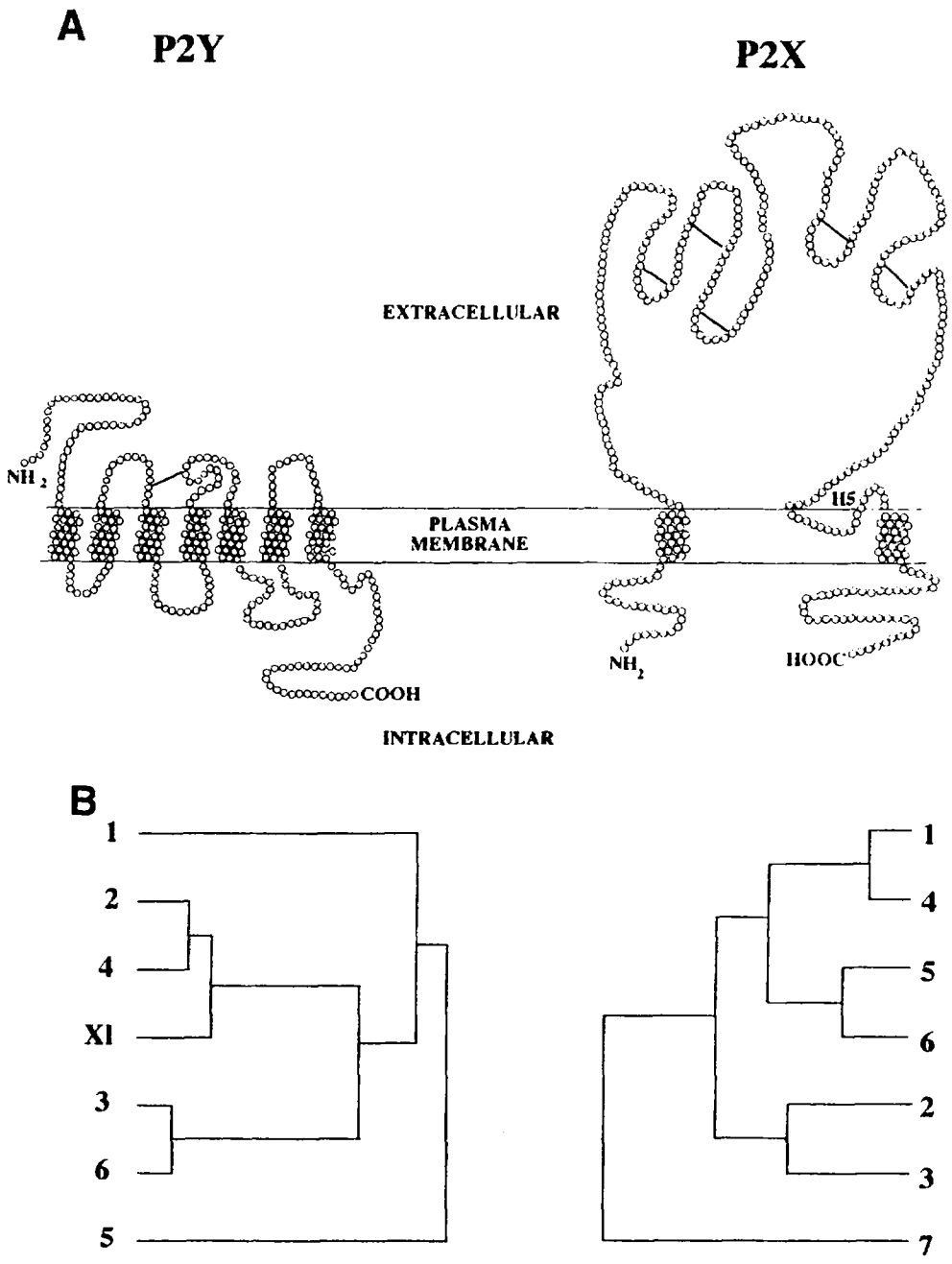


Fig. 1. **(A)** Predicted transmembrane topologies of the P2Y and the P2X receptors. Bars indicate predicted disulfide bridges. In the P2X structure, H5 is a strongly hydrophobic segment that has the highest degree of conservation among all the P2X receptor sequences (Fig. 4). It is indicated as a possible equivalent to the H5 (or "P loop") region of voltage-gated ion channels and of several transmitter-gated ion channels (Barnard et al., 1996). It has only slight sequence identity with any of these, but it may be associated with part of the TM domains and channel function, possibly in a re-entrant membrane topology (as for the known P-loops), as is speculatively shown. **(B)** Dendrograms representing structural relatedness within the two P2 receptor families. The plots were obtained from the multiple sequence alignment program CLUSTAL (Higgins and Sharp, 1988). The $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are of human origin, $P2Y_3$ and $p2y_5$ are from chicken, and X1-P2Y was isolated from *Xenopus*. All sequences in the P2X dendrogram are of rat origin.

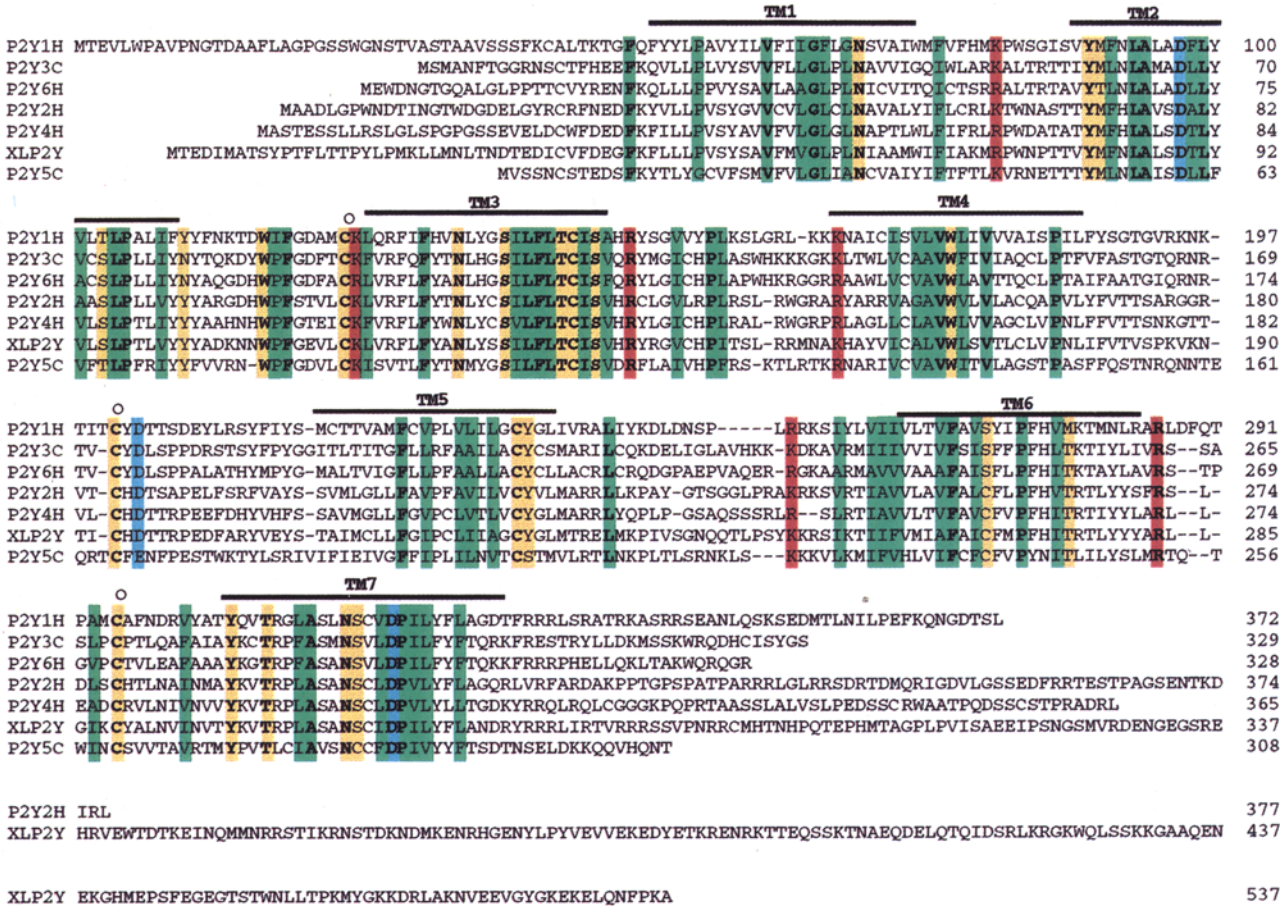


Fig. 2. Alignment of the P2Y receptor amino acid sequences. The predicted transmembrane domains are overlined. Conserved residues are indicated in bold. Conservation of residue type is indicated in color (blue, acidic; red, basic; yellow, polar; and green, hydrophobic). Conserved cysteine residues are indicated (O). Sequences where functional or binding evidence indicates membership of the P2Y receptor family are included. Species origins: human (H), chicken (C), and Xenopus (X).

nist, whereas pyrimidine based molecules and α,β-meATP were inactive. 2'-deoxyATPαS (dATPαS), a partial agonist, was found to be a very suitable ligand (^{35}S-labeled, K_D 7 n*M*) to determine receptor ligand affinities in membranes of COS-7 cells expressing this receptor (Simon et al., 1995a).

By cross-hybridization and PCR amplification based upon this clone, $P2Y_1$ receptors have been subsequently cloned from a variety of species (Table 1). The agonist profile obtained for the expressed bovine and human $P2Y_1$ receptors is broadly similar to that of chicken $P2Y_1$, with 2MeS-ATP apparently extremely potent (EC_{50} approx 40 n*M*; Henderson et al., 1995; Schachter et al., 1996). The P2Y antagonists suramin, reactive blue 2 (RB-2), and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) are strongly inhibitory at $P2Y_1$ receptors (Simon et al., 1995a; Charlton et al., 1996a).

As discussed below, the purity of nucleotides, presence of ectoenzymes, and nucleotide stability can all have an effect on the pharmacological profile observed for either a native receptor or one expressed heterologously. Indeed, it has recently been reported where special precautions are taken that ATP

is in reality an antagonist at the human $P2Y_1$ receptor (León et al., 1997). Further studies are therefore needed to determine the true pharmacological profile of this receptor.

The avian $P2Y_1$ receptor is expressed strongly in the brain, gut, lung, skeletal muscle, and less so in the spleen, stomach, and spinal cord (Webb et al., 1993; Filtz et al., 1994). The expression of the mammal receptor is more widespread, with the mRNA detected in most of the above tissues as well as heart, kidney, liver, placenta and pancreas (Tokuyama et al., 1995; Ayyanathan et al., 1996b).

The $P2Y_2$ / $P2Y_4$ / Xl-P2Y Receptor Subfamily

The mouse $P2Y_2$ receptor cDNA was also isolated in 1993 by Lustig and coworkers using a functional expression cloning strategy (Lustig et al., 1993). The $P2Y_2$ protein (Figs. 1, 2, and 5) resembles in activity a classical "P_{2U}" receptor, a subtype described on the basis of tissue responses to UTP as well as ATP (O'Connor et al., 1991). UTP, ATP, and Ap_4A are full agonists at this receptor type whereas 2-MeSATP is inactive (Lustig et al., 1993, Lazarowski et al., 1995). Under conditions in which nucleotide stability is ensured, ADP and UDP have no activity at this receptor type (Nicholas et al., 1996). $P2Y_2$ receptors with the same properties have been cloned from other species (Table 1). Where tested, their activation stimulates PLC leading to the mobilization of intracellular Ca^{2+}. These effects are antagonized by suramin, with a pA_2 value of 4.32, but to a lesser extent than at the $P2Y_1$ receptor (pA_2 = 5.77) whereas PPADS was ineffective (Charlton et al., 1996a). The $P2Y_2$ receptor transcript also has a wide tissue distribution and is present in spleen, kidney, liver, skeletal muscle and heart at high levels in comparison to the levels detected in the lung and brain (Lustig et al., 1993; Parr et al., 1994)

$P2Y_4$ was cloned from human genomic DNA and from placental and pancreas RNA (Communi et al., 1995; Nguyen et al., 1995; Stam et al., 1996). Analysis of the nucleotide efficacy at this receptor type originally indicated that UTP and UDP were full agonists with EC_{50} values of 2 μM (Communi et al., 1995) or 0.2 μM and 14 μM, respectively (Nguyen et al., 1995); whereas ADP and ATP were found to be weak partial agonists at best. Again, when the effects of the nucleotides were observed under stringent methodological conditions, UTP was a full agonist with an $K_{0.5}$ of 0.8 μM while UDP was found to be inactive. In addition, ATP acted as a full agonist although with lower potency than UTP with a $K_{0.5}$ of 39 μM (Nicholas et al., 1996). $P2Y_4$ receptors have been reported to be insensitive to suramin, but the effect of PPADS is unclear, as this has been found to be either inactive or active as an antagonist in two separate laboratories (Charlton et al., 1996b; Communi et al., 1996a). RB-2 has been reported to have some antagonist activity (Communi et al., 1996a). The $P2Y_4$ transcript has a more limited expression pattern; to date it has only been detected in placenta and pancreas in humans (Communi et al., 1995; Stam et al., 1996).

Bogdanov et al. (1997) have cloned a further cDNA member of this sequence subfamily from *Xenopus*. This sequence encodes a protein with an extended C-terminal tail of 216 amino acids in comparison with $P2Y_{1-6}$, which have comparatively short C-terminal cytoplasmic extensions of 15–65 amino acids. This receptor sequence has been named Xl-P2Y. Its pharmacological specificity was assessed by expression in *Xenopus* oocytes where it was found to recognises all endogenous nucleotides; an EC_{50} value of 100 nM was found for ATP, while 2MeS-ATP and α,β-meATP were both inactive. The nature of the response was also different from that of other P2Y receptors that have been characterized using this expression system. It was oscillatory upon application of low concentrations of ATP but was biphasic at higher concentrations, with the second phase persisting for up to 60 min after agonist removal. Suramin antagonized the membrane conductivity evoked by receptor activation, with an IC_{50} of 27 μM (Bogdanov et al., 1997). The *Xenopus* X1-P2Y receptor transcript is expressed transiently during embryonic development localized to the neural plate (Bogdanov et al., 1997).

Pharmacologically, the consistent feature of this receptor subfamily is that the triphosphates ATP and UTP have full agonist activity, dinucleotides have lower activity or are inactive, and 2-MeSATP is inactive as an agonist.

The $P2Y_3$ / $P2Y_6$ Receptor Subfamily

A further receptor cDNA was cloned from chick brain ($P2Y_3$), with a protein sequence that shares a similar degree of sequence identity with both the $P2Y_1$ and $P2Y_2$-like sequences. It responds to ADP (EC_{50} = 1.7 μM) and other nucleotides, both in oocyte expression and by Ca^{2+} mobilization in transfected mammalian cells (Webb et al., 1996b). $P2Y_3$ shows a preference for nucleoside diphosphates, with ATP and 2MeS-ATP relatively weakly active. Both RB-2 and suramin antagonize nucleotide responses at this receptor, with 100 μM suramin causing a 25-fold shift in the UDP EC_{50} value (Webb et al., 1996b).

The $P2Y_6$ receptor was originally cloned from rat aorta (Chang et al., 1995) and subsequently a human sequence has been isolated from placenta (Communi et al., 1996b). This receptor type has been shown to couple to phospholipase C. The agonist series for rat $P2Y_6$ was originally found to be UTP > 2-MeSATP, ADP >> ATP (Chang et al., 1995). UDP, which was not tested in the initial characterization, has greater efficacy than UTP, EC_{50} values of 0.1 and 1 μM respectively (Y. Takuwa, personal communication). The same rank order of activity was found for the human $P2Y_6$ receptor. Re-examination by Nicholas et al. (1996) indicated that the rank order of activity at the rat receptor was correct but that there was an underestimation of the difference in the activity of UTP vs UDP; pure UTP being 100-fold less potent than UDP. Furthermore, in this later study 2MeS-ATP and ATP showed considerably less activity. The former point can be attributed to nucleotide purity whereas the latter discrepancy awaits investigation. Thus, the true efficacy of these two ligands at this receptor is still in dispute.

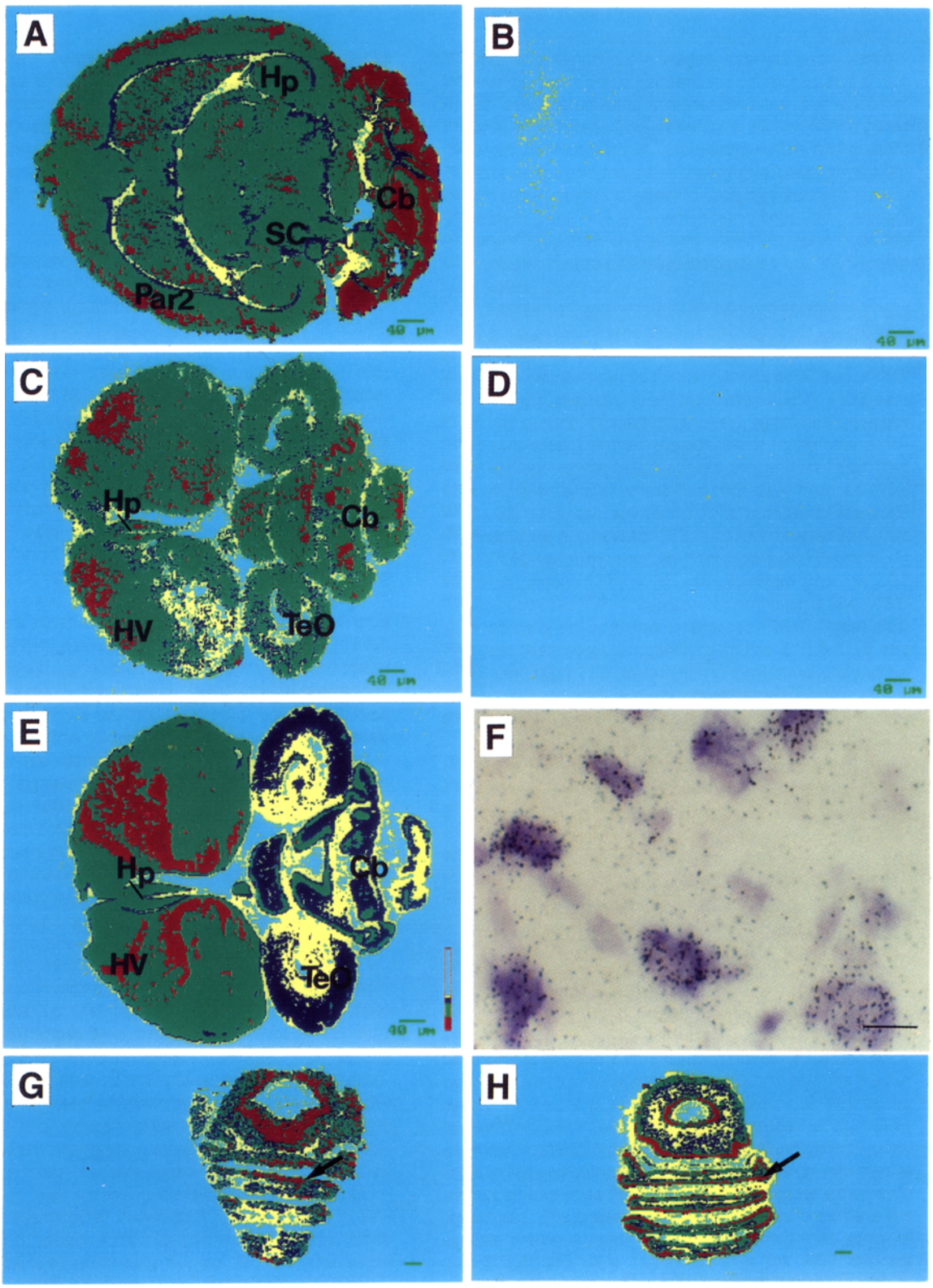
The $P2Y_3$ and $P2Y_6$ receptors share a high degree of sequence identity (60%) and have a very similar pharmacological profile. However, this degree of sequence identity is rather low for these receptor types to be assumed to be species homologs. Furthermore, they have differing tissue distributions as determined by Northern hybridization. The $P2Y_3$ mRNA was abundantly expressed in chicken spleen and was also expressed in brain, spinal cord, kidney and lung at lower levels (Webb et al., 1996b). The $P2Y_6$ message was detected in lung, digestive tract, spleen, and mesentery at high levels and was less abundant in the heart but was not detected in brain by Northern hybridization (Chang et al., 1995).

The $p2y_5$ Receptor

Webb et al. (1996c) noted the homology to P2Y receptor sequences of a chicken cDNA sequence (6H1; Kaplan et al., 1993) that would encode a 308-residue 7-TM protein. Webb et al. (1996c) expressed this receptor in COS-7 cells and found a high level of expressed binding sites for [^{35}S] dATPαS and adenine nucleotides, with the affinity order ATP > 2-MeSATP >> UTP. This was proposed as a new P2Y receptor subtype, $p2y_5$ (the lower case denotes that functional expression has not yet been investigated). The $p2y_5$ mRNA is absent from all avian tissues tested except T-cells, where it is present only upon their activation (Kaplan et al., 1993).

It should be noted that a $p2y_5$-like human sequence (also called $p2y_9$) that shares 61% amino acid identity with the chick sequence has also been isolated (Janssens et al., 1997). This receptor sequence was expressed in a number of cell lines and assayed for responses to a range of applied nucleotides. In no case was a positive effect noted for IP_3 generation, stimulation, or inhibition of adenylate cyclase or an acidification assay (Janssens et al., 1997). These authors concluded that this $p2y_5$-like sequence was not in fact a P2Y receptor. If this is the case this still does not exclude $p2y_5$, because another, partial human DNA sequence is now in Genbank that is more homologous to $p2y_5$ that is $p2y_9$.

A further sequence, named $p2y_{10}$, has been deposited at the EMBL database (Table 1) that


A
Hp
Cb
SC
Par2
40 µm
B
40 µm
C
Hp
Cb
HV
TeO
40 µm
D
40 µm
E
Hp
Cb
HV
Te
40 µm
F
G
H

shares identity with $p2y_5$. However, this assignment is also tentative as there is no functional data to support its inclusion in this receptor family.

A Related ATP-Binding Protein

A further cDNA was cloned from a human erythroleukemic cell line, which encodes a protein related in sequence to the other P2Y receptor subtypes (Akbar et al., 1996). In transfected cell expression this protein binds nucleotides with high affinity: the affinity for ATP was twofold higher than for 2MeS-ATP, with β,γ-meATP displaying greater affinity than at other P2Y receptor types and with UTP having much lower affinity than ATP. In fact, it contains a consensus sequence for a phosphate-binding loop (P loop) (GXXXXGK: Saraste et al., 1990), having the sequence GLGLVGK in its third extracellular loop. This protein was at first proposed as a further P2Y receptor, $P2Y_7$, but this assignment is not now made, since it has since been shown that it acts as a receptor for leukotriene B4 (Yokomizo et al., 1997).

This protein gave rise to a twofold increase in IP_3 above basal level in transfected cells stimulated with a saturating level of ATP (Akbar et al., 1996), but this limited response could perhaps be caused by a synergistic effect of the ATP binding on the response to low levels of endogenously released leukotriene. Although clearly not a true P2Y receptor, the functional effect of nucleotides on this protein requires fuller investigation.

True Pharmacological Profiles

K. Harden and coworkers (Nicholas et al., 1996) and Kennedy and Leff (1995) have introduced useful correctives for some of the previously reported agonist activity profiles for recombinant P2 receptors. Errors can occur because:

1. Contaminating nucleotides in commercial nucleotides (e.g., UDP in UTP) have given some erroneous series (e.g., if UTP has low activity at the receptor in question but UDP is active, as is the case for $P2Y_6$).
2. Hydrolysis by ectoenzymes can change nucleotide concentrations (and hence potencies) and species present, for example, the effect of UDP and ADP at $P2Y_2$ (Lazarowski et al., 1995).
3. An ectokinase on many cells can convert dinucleotides to trinucleotides, making assessment of UDP and ADP activities a

Fig. 3. *(previous page)* Distribution of the $P2Y_1$ receptor protein and its transcript in the rat and the avian brain. **(A–D)** Computer-generated pseudocolor images of representative adult rat (A, B) and 1-d-old chick (C, D) brain horizontal sections labeled by [^{35}S]dATPαS (*see* Simon et al., 1997b and Webb et al., 1997 for details). **(E, F)** *In situ* hybridization of the chick $P2Y_1$ receptor mRNA at macroscopic and cellular resolution, respectively Adjacent rat sections (14 μm) were incubated with: 10 n*M* [^{35}S]dATPαS alone (A) and (B) in the presence of 100 μ*M* 2MeS-ATP. Adjacent chick sections (14 μm) were incubated with 10 n*M* [^{35}S]dATPαS alone (C) and (D) in the presence of 10 μ*M* 2MeS-ATP. Note that the plane of section here is low in the chick compared to the rat, so that only the extremity of the Purkinje cell layer (red on the extreme right of C) is present, but **(G)** and **(H)** show a medial chick plane. Because of the high density of sites in the 1-d-old chick brain, to remain within a linear range the exposure time for (C) and (D) was 6 h compared to 16 h for (A) and (B) (but at the same specific activity of the ligand). (E) A parallel chick brain section to that used in (C) was hybridized with a mixture of three [^{35}S]-labeled antisense oligonucleotide probes specific for the chick $P2Y_1$ receptor mRNA. Exposure time was 7 d. (F) Cellular localization of the $P2Y_1$ receptor mRNA in the ventral hyperstriatum of the chick telencephalon. A bright-field photomicrograph of an emulsion-dipped section, counterstained with cresyl violet, is shown. (G) and (H) Parallel sections through the chick cerebellum showing the distribution of [^{35}S]dATPαS binding sites determined as in (C) and the localization of the $P2Y_1$ transcript (determined as in E), respectively. Note how the layering of the distribution is the same for both; the strongly positive sites are in the Purkinje cell and internal granule cell layers. Scale bars in (A–E) are 2 mm; in (F), 40 μm, and 0.7 μm in (G) and (H). For all panels except (F), autoradiographic film images were obtained after exposure of the sections to Hyperfim-βmax and were then analyzed with an image analysis system. Images were standardized using reference standards in each individual experiment in a linear response range. Red, green, blue, yellow, and cyan represent the highest through to nondetectable signal levels. Cb, cerebellum; Hp, hippocampus; HV, ventral hyperstriatum; Par2, parietal cortex area 2; SC, superior colliculus; and TeO, optic tectum.

problem. Again the $P2Y_2$ receptor is a good example (Lazarowski et al., 1995).

4. Nucleotides accumulating from cells can desensitize some P_2 receptors, for example responses to nucleotides were not detected in bovine $P2Y_1$ transfected Jurkat cells unless the cells were treated to remove released nucleotides (Henderson et al., 1995).

Effects 2 and 3 will, in fact, usually be minimized when very rapid measurements are made (e.g., for Ca^{2+} transients or fast-exchange patch-clamp recording) or with adequate flow rates of a large contact volume in oocyte recording; 4 is overcome by apyrase treatment. The effects of 2 and 3 will be important in the slow second messenger assays. In binding, they can be avoided by excluding Ca^{2+}, Mg^{2+}, and Na^+ (Simon et al., 1995b). It should be noted that apparent antagonist effects can also be modulated by the cell system used; suramin, reactive blue-2, and PPADS have all been shown to be inhibitors of ectoATPase (Chen et al., 1996).

Chromosomal Localization of P2Y Receptor Genes

The human $P2Y_1$ receptor has been localized to region q25 of chromosome 3 (Ayyanathan et al., 1996a). The human $P2Y_2$ gene has been located in region q13.5–14 of chromosome 11 (Dasari et al., 1996) whereas the human $P2Y_4$ gene has been mapped to chromosome Xq13 (Nguyen et al., 1995). None of these loci correlate with a disease state at present.

P2Y Receptors Expressed in the CNS and Their Methods of Signal Transduction

As noted above, a number of P2Y receptor subtypes have been detected in the brain by Northern hybridization. The distribution of the $P2Y_1$ receptor mRNA in the avian brain has been investigated by *in situ* hybridization (Webb et al., 1994, 1997). This receptor transcript was found to be abundant and exhibited a regional-specific pattern of expression throughout the brain, being present in the avian forebrain as well the midbrain and cerebellum (Fig. 3E). Analysis at the cellular level revealed that the mRNA is localized on many neuronal cell bodies but the presence of silver grains not localized over the stained cell bodies indicates that the transcript is also expressed by their fiber tracts and by glial cells as illustrated for the ventral hyperstriatum in Fig. 3F (Webb et al., 1997). To date the localization of other P2Y receptors within the CNS remains to be reported.

The macroscopic localization of the avian $P2Y_1$ mRNA corresponds essentially to the distribution of binding sites for [^{35}S]dATPαS which are sensitive to 2MeS-ATP (Fig. 3C, D). These have been localized in detail in the chick and rat brains, and display a pharmacological series in ligand displacement that corresponds to the affinity series of the $P2Y_1$ receptor (Webb et al., 1997; Simon et al., 1997b). It should be noted that this binding would include any P2Y receptor with a broadly similar specificity to $P2Y_1$. Furthermore, some topographically comparable structures in the avian and rodent brain exhibit comparable levels of [^{35}S]dATPαS binding sites (Fig. 3, Table 2), which indicates that the location of the $P2Y_1$ receptor in homologous areas has been preserved during evolution. It has recently been reported that this same radioligand gives very high binding to COS-7 cells that is not caused by P2 receptors (Schalcher and Harden, 1997). However, at the much higher concentration used there, in a range above 1 μ*M*, such nonspecific binding would be expected to be very high for most types of receptor-ligand binding. Furthermore, we have previously established saturable binding of [^{35}S]dATPαS in the range up to 125 n*M* (K_d: 7–13 n*M*), with an acceptably low nonspecific background, for both chick and rat brain membranes (Simon et al., 1995b).

Experiments on cultured cells and on brain slices have indicated that metabotropic nucleotide receptors are found in all cell types in the brain, i.e., astrocytes, oligodendrocytes, neu-

Table 2
Density of [^{35}S]dATPαS Binding Sites in Homologous Regions of the Avian and Rodent Brain

Structure	Density	Structure	Density
Avian		Rodent	
Striatal cortex	++[a]	Neocortex	+++
Hippocampus	++	Hippocampus	++
Advancedpalestriatum	++++	Caudate putamen	++++
Primative palestriatum	+++	Globus pallidus	+++
Lateral septal nucleus	+++	Lateral spetal nucleus	++
Lateral, paradorsal mesemcephalic Nucleus	++++	Inferior colliculus Nucleus	+++
Optic tectum	++	Superior colliculus	+++
Cerebellum	++	Cerebellum	++

[a]The density of [^{35}S]dATPαS binding sites were scored as (++) low, (+++) moderate, or (++++) strong and are equivalent to blue, green, and red respectively in Fig. 5. Data taken from Webb et al. (1997) and Simon et al. (1997).

rons, and microglia. Correspondingly, extracellular nucleotides have been shown to elicit the activation of a wide range of second messenger pathways in the CNS.

A classical mechanism associated with the metabotropic P2Y receptors is the activation of phospholipase C leading to mobilization of calcium (Kastritsis et al., 1992) and this is the recognized pathway of the $P2Y_1$ receptor in a number of locations in the brain. However, in a clonal line of blood–brain barrier endothelial cells (B10), activation of the $P2Y_1$ receptor was shown to elevate intracellular calcium levels by the inhibition of adenylate cyclase rather than activation of phospholipase C (Webb et al., 1996a). The inhibitory effects of extracellular nucleotide application on adenylate cyclase activity have also been well documented for the C6 glioma cell line (Boyer et al., 1993; Lin and Chuang et al., 1994) and may be because of the presence of the same rat $P2Y_1$ receptor as in the B10 cells (Webb et al., 1996a), or a related receptor with a similar pharmacological profile to the $P2Y_1$ type (Boyer et al., 1994; 1995).

Mature oligodendrocytes and their late glial precursors from cortex and retina express P2Y receptors of two types, the activation of which leads to intracellular calcium release by a thapsigargin- and heparin-sensitive mechanism. However, their early glial precursor cells do not respond to nucleotides (Kirischuk et al., 1995a).

Cultured astrocytes from the cerebellar cortex have also been found to express P2 receptors coupled to calcium mobilization (Langley and Pearce, 1996). One of the downstream consequences of their activation is the stimulation of mitogen-activated protein kinase activity, possibly via mitogen-activated protein kinase kinase activation that implicates nucleotide receptors in the regulation of cell proliferation, differentiation, and stress responses (Neary and Zhu, 1994). $P2Y_1$ receptor activation causes phospholipase C-mediated calcium mobilization in primary cultured astrocytes from the dorsal horn of the spinal cord (Salter and Hicks, 1995). Dorsal horn astrocytes also express a UTP-activated receptor sensitive to suramin and PPADS that mediates calcium mobilization by the same mechanism (Ho et al., 1995). Further, a subpopulation of neurons and oligodendrocytes from this location also exhibited calcium mobilization in response to ATP application (Salter and Hicks, 1994). In cerebellar slices, ATP caused release of intracellular calcium via IP_3 release in Bergmann glial cells (Kirischuk et al., 1995b). Responses were greater at the distal processes rather than the soma, indicating a nonuniformity in the expression of the receptors or in the downstream second messenger system.

P2Y receptors can also modulate ion-channel activity. Activation of the $P2Y_2$ receptor in

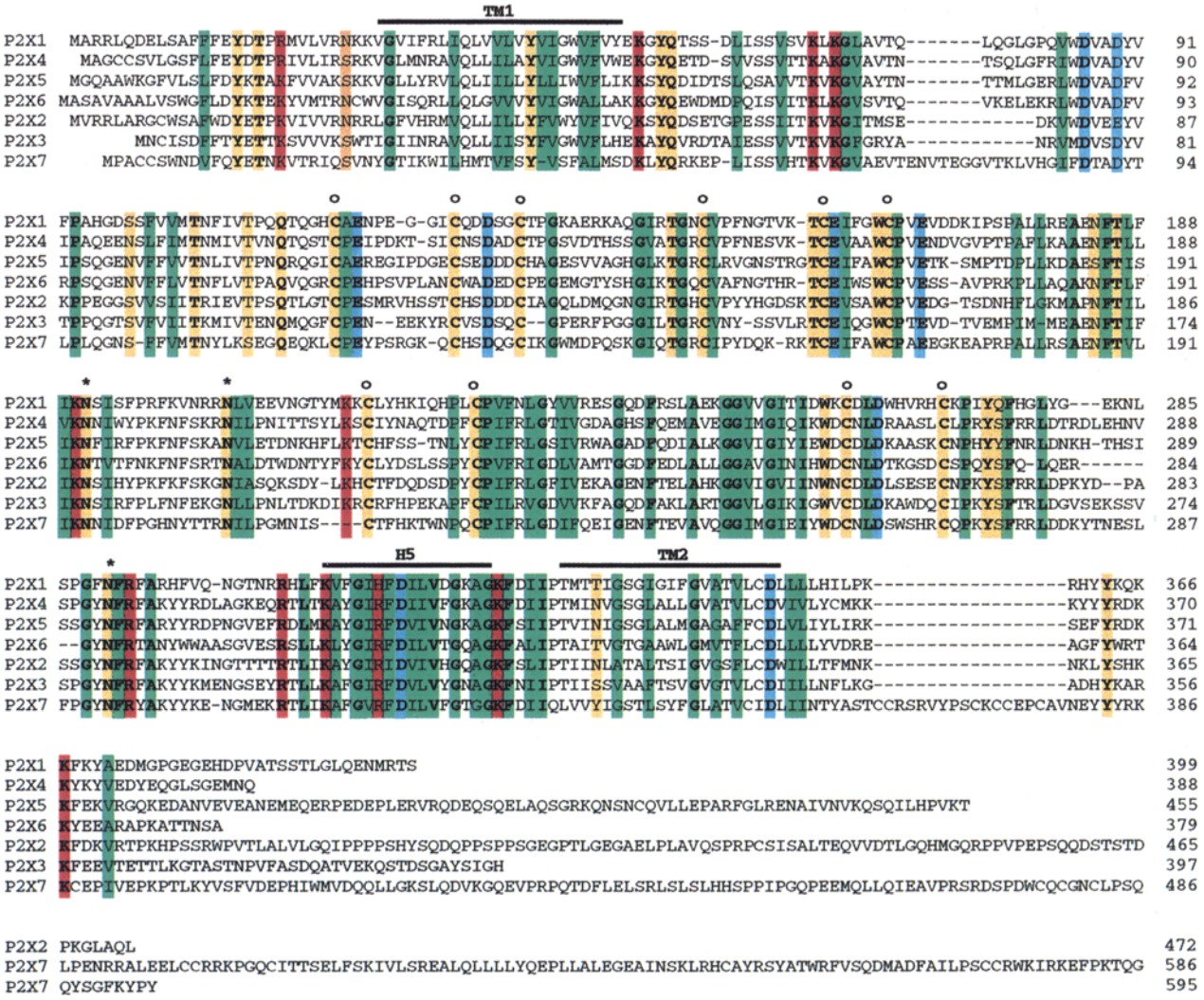


Fig. 4. Alignment of the amino acid sequences of the known P2X receptor subunits. The predicted transmembrane domains and H5-like region are overlined. Conserved residues are indicated in bold. Conservation of residue type is indicated in color (blue, acidic; red, basic; yellow, polar; and green, hydrophobic). Conserved cysteine residues (○) and putative asparagine-linked glycosylation sites (*) are also indicated. Alternate forms of the $P2X_2$ subunit produced by alternate splicing are not included. All sequences are of rat origin.

the NG108-15 neuroblastoma/glial cell hybrid has been shown to inhibit the voltage-gated "M current" K^+ channel via a perussis toxin (PTX)-insensitive G protein (Filippov et al., 1994). P2Y receptor modulation of N- and L-type Ca^{2+} channels in this same cell line has also been characterized (Filippov and Brown, 1996). N- and L-current inhibitions were mediated by two different G proteins, since the N-type channel was sensitive to PTX and the effect at the L-current was not (Filippov and Brown, 1996). The K^+ channel and Ca^{2+} channel inhibition in NG108-15 cells are contributed by different receptors, based on the rank order of potency of the nucleotides tested at both responses. Indeed, the latter effect has been attributed partly to the $P2Y_2$ receptor, which has been shown to inhibit the N type Ca^{2+} channel in a recombinant assay system based on microinjection of neurons (Filippov et al., 1997).

The $P2Y_1$ receptor has been implicated in the activation of an outwardly rectifying K^+ channel in a range of primary cultured rat neurons, by subtly different mechanisms involving a PTX-insensitive G protein. Activation of that K^+ channel in inferior collicular and cerebellar neurons by nucleotides is rapid, with the onset for the response <1 s. The evidence suggests that this occurs upstream of phospholipase A2 or protein kinase C (PKC) activation, indicating a direct modulation of channel activity by the G protein subunits (Ikeuchi and Nishizaki, 1995, 1996). Activation of this same current in other cultured neurons has been found to be mediated downstream by PKC activation; in hippocampal neurons this effect is dependent on PKC (Ikeuchi et al., 1996b), whereas in superior colliculus neurons it appears to be independent of both phospholipase C and phospholipase A_2 activation suggesting a novel PKC activation pathway in these cells (Nishizaki and Ikeuchi, 1996). A latency in the response to receptor activation achieved by these mechanisms of K^+-channel activation would provide a means for fine tuning the inhibitory transmission in neurons.

Channel modulation has also been found in nonneuronal cells in the brain. In the macrophage-like microglia 2MeS-ATP evokes an outwardly rectifying K^+ current via a PTX-sensitive G protein (Langosch et al., 1994). Microglia also possess P2X receptors, including the permeability receptor $P2X_7$ (as discussed below).

Functional Significance of CNS P2Y Receptors

As in other tissues, nucleotides act as cell-to-cell signalers in the brain. Nucleotides have also been noted as having mitogenic and morphogenetic properties in the nervous system (Neary et al., 1996) and may be mediators of astrocyte responses to injury. The latter function would be similar to the one for which evidence has been gathered in peripheral tissues, where P2Y receptors are believed to often act as monitors of injury to neighboring cells: the release of ATP (which is at a high concentration in all active cells) is an early consequence of cell damage or deformation and evokes a reaction, including cytokine release, in the surrounding cells (Schlosser et al., 1996; Wang et al., 1996b). Similar leakage of UTP and the cleavage of both ATP and UTP to their dinucleotides by ubiquitous ectonucleotidases, means that P2Y subtypes, where those components are active, are also involved. A related process has been found to contribute to the molecular basis of touch sensitivity. ATP can be released by deformation from the terminals of the sensory nerves involved to initiate sensory transmission via $P2Y_1$ receptors (Nakamura and Strittmatter, 1996).

P2Y receptors can, in some locations, act directly in neurotransmission (Illes et al., 1997). Thus, they have been implicated in increasing the spontaneous firing rate in a subset of neurons of the rat medial vestibular nucleus (Chessell et al., 1997). Adenine nucleotides increase the firing rates of locus coeruleus neurons in brain slices, whereas UTP is inactive (Frolich et al., 1996). The P2-receptor antagonists suramin, reactive blue-2, and PPADS have been shown to block glutamatergic transmission in CA1 and CA3 pyramidal neurons (Motin and Bennett, 1995). The effects of suramin and reactive blue-2 (but not PPADS) were in part postsynaptic, as their application blocked the effect of applied glutamate. However, P2X and P2Y effects may not be discriminated clearly by these antagonists; indeed the effects of ATP on various neurons are likely to be via both P2Y and P2X receptors. Regardless of the receptor type involved, the ineffectiveness of PPADS in blocking the glutamate effect may indicate a presynaptic action of ATP on glutamate release. ATP has been shown to induce long-term potentiation in pyramidal neurons (Wierasko and Ehrich, 1994) and has been found to increase intracellular calcium via metabotropic nucleotide receptors in a subpopulation of dissociated hippocampal neurons (Mironov, 1994). At the neuromuscular junction, an elegant recent study (Silinsky and Redman, 1996) has shown that ATP is released

absolutely synchronously with acetylcholine and may serve to limit transmitter release.

The expression of P2Y receptors on the distal processes of Bergmann glial cells could be functionally important, because of the intimate interaction of the Bergmann glia processes with the Purkinje cell dendritic tree. It has been speculated that ATP released from the synaptic ending of parallel and climbing fibers, which innervate Pukinje neurons, may generate calcium responses in Bergmann glial cells and lead to alteration of their intracellular calcium concentration and possible efflux of calcium into the extracellular space, this is of potential importance as it has been shown that the extracellular concentration of this ion can be depleted during electrical stimulation (Kraig and Nicholson, 1978; Benninger et al., 1980).

In summary, the neuronal P2Y receptors may be either excitatory or inhibitory depending on their location and their coupling to different transduction systems. Modulation of ion-channel activity, in particular at Ca^{2+} currents, by P2Y receptors represents a mechanism of transmitter release regulation, whereas the inhibition of M-currents provides a mechanism for neuronal excitation.

The P2X Receptors

Recent cloning of cDNAs for the P2X receptor have justified a new family of the transmitter-gated ion channels for these receptors. They are structurally distinct from all other known transmitter-gated channels such as those for acetylcholine and glutamate (Surprenant et al., 1995). Seven subunits of the P2X receptor have been identified to date by molecular cloning (North and Barnard, 1997). They share an overall sequence identity ranging from 27 to 50% and their sizes are ranging from 388 to 595 amino acids (Figs. 1, 4).

Their main structural characteristics are two transmembrane domains (TM1 and TM2) separated by a long loop segment (of approx 300 amino acids), and relatively short N- and C-termini, with the exception of $P2X_2$ and $P2X_7$, which have an extended C-terminal tail (Fig. 4). The P2X subunits show similarity in this topology (but not in sequence) to a distinct class of ion channels with two transmembrane domains, which includes the amiloride-sensitive epithelial sodium channels (ENaC), the inward-rectifier potassium channels, the Phe-Met-Arg-Phe-NH_2 (FMRF)-gated channel and the mechano-sensitive channel of *Escherichia coli* (reviewed by North, 1996). Only in the ENaC type is the long extracellular loop also found. The P2X receptor family, rather, is placed in the context of transmitter-gated channels (Barnard, 1996). In addition, a highly homologous hydrophobic segment (H5), precedes the second transmembrane domain is in each P2X subunit (Figs. 1, 4). This region together with the second transmembrane domain is proposed to form the ionic pore. The TM1–TM2 loop contains 66 completely conserved residues, located in a number of separate blocks. These include 10 cysteines, 13 glycines, 6 lysines, 4 prolines, and 3–6 possible sites for N-linked glycosylation (Fig. 4). This loop is presumed to be extracellular because it can be glycosylated in an in vitro translation system (in the $P2X_1$ and the $P2X_2$ receptor, Valera et al., 1995; Simon et al., 1997a) and because it contains a lysine residue which introduces sensitivity to externally applied PPADS that could form a Schiff base there (Buell et al., 1996). The size of the glycosylated product (60 kDa) is very similar to that found for a P2X receptor protein that was partially purified from rat vas deferens and photoaffinty labelled by [^{3}H]α,β-meATP (Bo et al., 1992). The conserved cysteines could form a network of disulfide bonds to provide a compact tertiary structure and generate the binding site for ATP. Although there are highly conserved motifs, such as XGGXXG, in the extracellular loop or XGXXGKF in H5 region (Fig. 4) that might suggest consensuses for ATP binding sites, none of them conforms even to the more generalized phosphate-binding motif GXXXXGK, which is common but not universal in all adenine and guanine nucleotide-binding

proteins (Saraste et al., 1990). The C-terminal tail region is the least conserved in all the subunits.

A longer C-terminal tail found for the $P2X_2$ and $P2X_7$ subunits might be responsible for important functions such as the nondesensitizing character of the $P2X_2$ channel or formation of large pores of the $P2X_7$ channel (Brake et al., 1994; Surprenant et al., 1996). Indeed, the C-terminal tail of the $P2X_2$ subunits contains two proline-rich segments similar to those found in a number of transmembrane proteins including the epithelial sodium channel (αrENaC) and the NMDA receptor NR2D subunit. These domains could form complexes with the SH3 domain of certain intracellular signaling and cytoskeletal proteins such as *src*-like tyrosine kinases and α-spectrin (Rotin et al., 1994). Absence or presence of these very hydrophobic fragments may introduce fine control of the desensitization of this channel (Simon et al., 1997a).

Pharmacological Properties of the P2X Channels

Each of the known P2X subunits can form a functional homo-oligomer channel when they are expressed heterologously in mammalian cells or *Xenopus* oocytes. However, the stochiometry of the channels formed thus is not known. The distinction among the different homo-oligomeric channels formed by the seven known P2X subunits have focused on four pharmacological phenomena; relative sensitivity to α,β-meATP (*see* Introduction), sensitivity to P2 antagonists (suramin and PPADS), fast or slow desensitization of agonist responses, formation of a large ionic pore (*see* Fig. 5). The latter characteristic is attributed only to a P2 receptor earlier recognized as permeabilizing certain cells of the immune system and known as the P2Z receptor. Since molecular cloning of cDNA for this receptor revealed its structural homology to the the known recombinant P2X receptors, this receptor became the $P2X_7$ receptor type.

Figure 5 summarizes the phenotypes of the seven P2X receptors now known, in terms of the pharmacological criteria as observed when expressed in *Xenopus* oocytes or mammalian cells. Thes phenotypic distinctions, as with the P2Y receptors, groups, do not always correlate with the structural relatedness of the different P2X subunits displayed in the dendogram of Fig. 1.

$P2X_1$ and $P2X_3$ Receptors

These two P2X subunits represent the first phenotype. The $P2X_1$ subunit was the first recombinant P2X receptor to be identified, by expression cloning from rat vas deferens (Valera et al., 1994). Recently, species orthologs of this subunit were isolated from mouse and human urinary bladder (Valera et al., 1995). Large inward currents to ATP, α,β-meATP, and 2MeS-ATP were seen when the recombinant $P2X_1$ receptor was expressed in *Xenopus* oocyes or heterologously in HEK 293 cells (Valera et al., 1994; 1995). These currents desensitized very rapidly. 2MeS-ATP is a more potent agonist at this recombinant receptor than α,β-meATP. The rank order of potency of different P2 agonists are 2MeS-ATP≥ATP>α,β-meATP>>ADP. It should be noted that α,β-meATP is a much more resitant ligand to ectonucleotidases then the other agonists (Welford et al., 1987), so its lower activity here is significant.

In contrast, in ligand-binding studies with [^{35}S]-ATPγS (previously shown to label P2X binding sites; Michel et al., 1996a) or [^{3}H]α,β-meATP on the human recombinant $P2X_1$ receptor transiently expressed in Chinese hampster ovary (CHO-K1) cells, α,β-meATP possessed relatively low affinity when divalent cations were absent (Michel et al., 1996b). Inclusion of 4 m*M* Ca^{2+} greatly increased the affinity of α,β-meATP as was also observed in rat vas deferens membranes expressing the native $P2X_1$ receptor (Michel and Humphrey, 1994). It is not known whether this Ca^{2+} dependence of the high-affinity α,β-meATP binding is a direct effect of Ca^{2+} on the receptor protein or because of its binding to the ligand. The P2 antagonists, suramin and PPADS readily antagonize the agonist-evoked responses. This type of response has also been found in many native tissues such as smooth muscle (Benham and Tsien, 1987), sensory and sympathetic ganglia (Kristhal et al., 1988; Evans et al., 1992; Khakh et al., 1995; Surprenant et al., 1995),

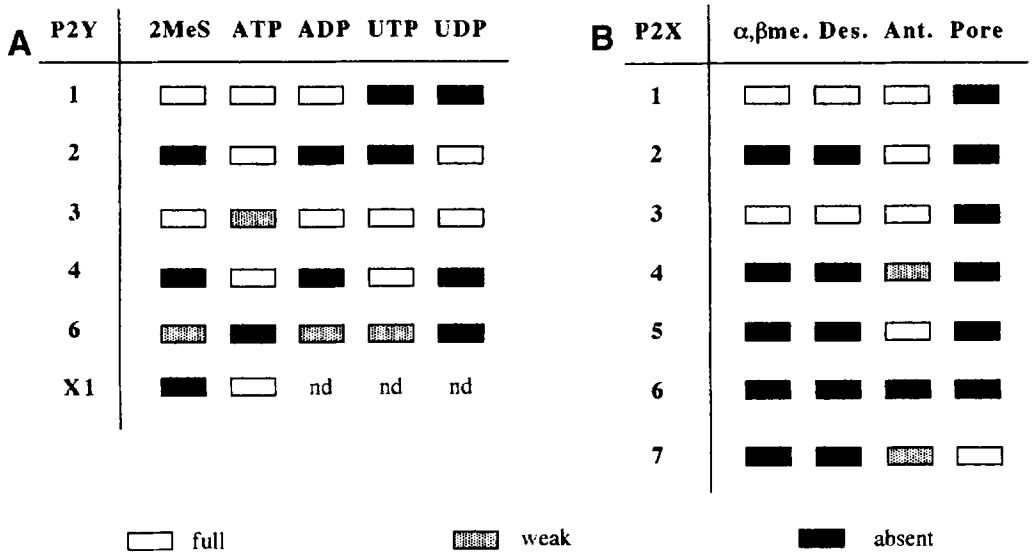


Fig. 5. Schematic diagrams representing the major differences in properties among the P2Y **(A)** and P2X **(B)** receptor phenotypes. For the P2Y receptors, sensitivities to the indicated nucleotides are compared (2MeS = 2MeS-ATP). For the homomeric P2X channels, four diagnostic properties are noted: (1) agonist sensitivity to α,βme-ATP (α,βme.); (2) rate of desensitization upon continued agonist application (Des.); (3) sensitivity to P2 receptor antagonists (Ant.); and (4) ability to form a large membrane pore on repeated agonist application (pore). "Full" indicates a high potency, rapid desensitization, or formation of the large pore. Desensitization and pore formation are rated only for presence or absence. 2MeS-ATP and ATP in (A) may include the corresponding diphosphate, for $P2Y_1$.

and medial habenula of rat brain (Edwards et al., 1992).

A cDNA for the $P2X_3$ subunit was cloned from rat dorsal root ganglia (Chen et al., 1995; Lewis et al., 1995). This recombinant receptor has similar pharmacological properties in terms of agonist selectivity, rate of desensitization and antagonism to that of the $P2X_1$ subtype. Currents evoked by agonists (in the potency order of 2MeS-ATP≥ATP≥α,β-meATP>ATPγS>>ADP) showed even faster desensitization than that of the $P2X_1$ channel (in a range of milliseconds) and were blocked by suramin or PPADS (EC_{50}: 1–3 μ*M*). This receptor has a very limited distribution, only detected as yet on sensory neurones (Chen et al., 1995; Lewis et al, 1995).

$P2X_2$ and $P2X_5$ Receptors

The $P2X_2$ receptor cDNA was obtained from rat P12 pheochromocytoma cells by expression cloning in *Xenopus* oocytes (Brake et al., 1994). Agonists, such as ATP≥2MeS-ATP≥ATPγS, are usually 20–30 times less potent at this receptor than at the $P2X_1$ (IC_{50}: approx 30 μ*M* vs approx 1 μ*M*) to evoke inward currents (Brake et al., 1994). α,β-meATP previously thought to distinguish the P2X from the P2Y types (Burnstock and Kennedy, 1985) has no agonist activity at this receptor. During prolonged application of agonist this channel shows very little desensitization with a time constant of 112 s (Brake et al., 1994; Simon et al., 1997a). In ligand-binding studies performed with [^{35}S]ATPγS, the recombinant $P2X_2$ displayed no difference from the recombinant $P2X_1$ receptor in terms of agonist selectivity when divalent cations were absent from the reaction. α,β-meATP bound extremely weakly and unlike at the $P2X_1$ receptor addition of Ca^{2+} failed to increase the affinity of this ligand (Michel et al., 1996c). Suramin and PPADS are, as it was found for the $P2X_1$ receptor, potent antagonists. However, there is

some difference in the antagonist potencies: PPADS, DIDS, and dextran sulfate were 7–30 times more potent in inhibiting [^{35}S]ATPγS binding to the recombinant human $P2X_1$ receptor than to the $P2X_2$, when both are heterologously expressed in CHO-K1 cells (Michel et al., 1996c).

A cDNA encoding the $P2X_5$ subunit was cloned from rat celiac ganglia by Collo et al. (1996). This receptor is also able to form a functional homo-oligomer channel and has very similar pharmacological properties in terms of agonist potency (ATPγS,>ATP=2MeS-ATP >>ADP), antagonist sensitivity, and desensitization to the $P2X_2$ channel, when they both are heterologously expressed in human embryonic kidney (HEK 293) cells. However, the $P2X_5$ subunit has a restricted tissue distribution (Garcia-Guzman et al., 1996). This subtype also shows, like $P2X_2$, a lack of α,β-meATP sensitivity, and minimal desensitization (Fig. 5). Another major difference from the other known subtypes of the P2X receptor is that the agonist-evoked peak current for the $P2X_5$ homo-oligomer channel was always very small, being 10-fold less than any other recombinant P2X subtype expressed heterologously in HEK 293 cells (Collo et al., 1996).

$P2X_4$ and $P2X_6$ Receptors

cDNAs for the $P2X_4$ and the $P2X_6$ subtypes were isolated from rat cervical superior ganglia by G. Buell and his colleagues in Geneva (Buell et al., 1996; Collo et al., 1996). Simultaneously, a cDNA was obtained from rat brain for $P2X_4$ by Bo et al. (1995). cDNAs encoding the $P2X_4$ subunit were also isolated by others (Seguela et al., 1996; Soto et al., 1996a) from rat brain, and more recently from human brain (Garcia-Guzman et al., 1997). The $P2X_6$ cDNA was also cloned by Soto et al. (1996b) from rat brain. These subunits can also form functional homo-oligomeric channels, although the $P2X_6$ channel expresses very poorly both in *Xenopus* oocytes and mammalian cell lines, suggesting that this subunit may require a partner subunit for full expression (Collo et al., 1996). Again, α,β-meATP has no or very weak activity on these channels (Fig. 5). A very small current was observed for the $P2X_4$ homo-oligomeric channel with 100 mM α,β-meATP (Soto et al., 1996a) while at the $P2X_6$ channel this compound was completely inactive (Collo et al., 1996). Rank order of potency of P2 agonists are: $P2X_4$: ATP≥2MeS-ATP>ATPgS>>ADP>α,β-meATP; $P2X_6$: ATP≥2-ClATP≥2MeS-ATP> ATPγS>>ADP (α,β-meATP inactive).

Like the $P2X_2$ channel, neither of the $P2X_4$ or the $P2X_6$ channels showed apprecible desensitization during continued application of agonists (Buell et al., 1996; Collo et al., 1996). Moreover, the agonist-induced current at the $P2X_4$ channel can be potentiated by a low concentration of extracellular Zn^{2+} ions (Seguela et al., 1996; Garcia-Guzman et al., 1997) suggesting a role in Zn^{2+} induced neuronal hyperexcitability in the hypocampus where the mRNA for this subunit has been found abundant (Collo et al., 1996; Seguela et al., 1996). Unexpectedly, these channels were found to be resistant (Bo et al., 1995; Buell et al., 1996) or partially resistant ($P2X_4$ channel) (Seguela et al., 1996; Soto et al., 1996a, 1997) to antagonist blocade.

It has recently been shown by Buell et al. (1996) that a lysine residue (Lys^{249}) located in the extracellular loop is responsible for the slow reversible component of blockade of PPADS through formation of a Schiff's base with this residue. This lysine residue has been found in the $P2X_1$, $P2X_2$, and $P2X_5$, but not in the $P2X_4$ and $P2X_6$ subunits where it is substituted by glutamate and leucine, respectively (Fig. 4). Mutation of the glutamate or leucine residue to lysine renders both the $P2X_4$ and the $P2X_6$ receptor sensitive to PPADS (Buell et al., 1996). However, the action of suramin was not affected by the above mutations, suggesting that this compound interacts with the P2X receptors in a different fashion. Direct radioligand binding experiments were also performed on the recombinant rat $P2X_4$ receptor heterologously expressed in CHO-K1 cells. The binding of [^{35}S]ATPγS to the recombinant $P2X_4$ receptor was also weakly inhibited by

suramin, PPADS, and number of other P2 antagonists and this interaction was allosteric rather than competitive (Michel et al., 1997).

More recently a human homolog of the $P2X_4$ subunit has been identified with higher antagonist sensitivities (Garcia-Guzman et al., 1997). Domain exchange experiments between the human and rat isoform have identified a domain (81–183) located in the extracellular loop that could account for the higher PPADS sensitivity in the human form (Garcia-Guzman et al., 1997). Moreover, mutation experiments have given an explanation for the increased suramin sensitivity of the human the $P2X_4$ receptor. If a single residue, Gln^{78} in rat $P2X_4$ sequence is exchanged for lysine (normally present in the human sequence at this position) the suramin sensitivity of the mutated receptor increases by 50-fold (Garcia-Guzman et al., 1997). However, this lysine residue, present in the human $P2X_4$ is absent in the other suramin-sensitve P2X subtypes (Fig. 4), suggesting that channels formed by the different P2X subtypes use different mechanisms for binding suramin.

$P2X_7$ Receptor

This subtype was formerly classified as the P2Z type, thought to be extremely distinct from the P2X and P2Y types, based on its unusual pharmacological properties, including block by Mg^{2+} (Fredholm et al., 1994). ATP acting through this receptor permeabilizes cells and lyses macrophages by opening large membrane pores (Nuttle and Dubyak, 1994). However, this receptor has been identified by cDNA cloning as a member of the growing family of the P2X receptors (Surprenant et al., 1996).

The P2X channel formed by this subunit has a very distincive pharmacology. Brief application of agonist in the presence divalent cations opens the $P2X_7$ channel, permeable only to small cations like the other known P2X channels. However, removal of extracellular divalent cations and repeated application of agonist produces a much larger, sustained inward current and leads to the lysis of the cells, because of the formation of large membrane pores (Surprenant et al., 1996). The agonist pharmacology at this receptor is also unique among the P2 receptors, 3'-*O*-(4-benzoyl benzoyl) ATP (BzATP)>>ATP>2MeS-ATP>ATPγS>>ADP (α,β-meATP inactive). The current evoked at this channel desensitizes very slowly (>10 s) and is relatively insensitive to suramin and moderately sensitive to PPADS (IC_{50}: approx 50 μ*M*). More recently, the human form of the $P2X_7$ subunit has been obtained (Rassendren et al., 1997). The properties of the human receptor broadly resemble those of the rat. However, there are marked differences, i.e., a lower agonist potency with the EC_{50}s for BzATP and ATP 25- and 10-fold greater than for the $rP2X_7$ receptor, respectively.

The $P2X_7$ subunit has a C-terminal tail approx 200 amino acids longer than in any other known P2X subunits. Mutagenesis experiments have shown that this long C-terminal tail is responsible for the large pore formation, though it does not affect the receptor function as an ATP-gated nonselective cation channel (Surprenant et al., 1996). C-terminal domain exchange experiments between the human and the rat form of $P2X_7$ subunits have provided further insight into how the long C-terminal tail of the $P2X_7$ subunit controls the large pore formation (Rassendren et al., 1997).

Channel Assembly and Heteropolimerization

All seven P2X subunits can assemble as homo-oligomeric functional channels. However, the $P2X_5$ and $P2X_6$ channels express very weakly when their cDNAs are individually transfected into mammalian cells (Collo et al., 1996), suggesting that these subunits are more likely to heteropolymerize with other P2X subunits. It is common in other transmitter-gated channel families for different subunit types to assemble to form native functional receptors, as with nicotinic acetylcholine, glutamate, and $GABA_A$ receptors (Barnard, 1996). Homo-oligomer formation of a native-type receptor is

uncommon for the extracellularly gated receptor channels, however, being known for the 5-HT_3 receptor.

Heteropolymerization of P2X subunits has been demonstrated for the $P2X_2$ and $P2X_3$ so far (Lewis et al., 1995). These receptors, when expressed as homo-oligomers, have clearly different properties, the $P2X_2$ channel being insensitive to α,β-meATP and desensitizing very slowly, and the $P2X_3$ channel being sensitive to α,β-meATP and desensitizing rapidly (Fig. 5). Coexpression of the $P2X_2$ and $P2X_3$ subunits in mammalian cells produce a channel with mixed characteristics (α,β-meATP-sensitive and desensitizing). P2X currents displaying a similar phenotype have been recorded from some sensory ganglia (Khakh et al., 1995; Lewis et al., 1995), where both $P2X_2$ and $P2X_3$ mRNAs and proteins are expressed.

Such heteropolymerization of P2X subunits is expected to be more widespread for a number of reasons: mRNAs for all subunits are present in some sensory ganglia; $P2X_4$ and $P2X_6$ mRNAs show a strikingly widespread coexpression within the nervous system; and human genes for $P2X_1$ and $P2X_5$ and for $P2X_4$ and $P2X_7$ are clustered in close proximity on the same chromosome (discussed below). Coimmunoprecipitation studies with subtype specific antibodies are required to see whether heteropolymerization with P2X subunits other than $P2X_2$ and $P2X_3$ occurs in native cells and this approach might also provide information about the relative numbers of the subunits per channel.

Distribution of P2X Receptor Subtypes

Data on the distribution of the seven cloned P2X subunits are mainly based on Northern blotting and *in situ* hybridization studies but in the case of $P2X_1$-$P2X_3$ these have also been extended to the protein level by immunohistochemistry (Vulchanova et al., 1996; Cook et al., 1997). A wide variety of tissues and cell types, including smooth muscle, neuronal, and immune system cells, express at least one of the seven P2X subunits. In the nervous system, these receptors are in the central, autonomic, sensory, and enteric systems and also present on microglia.

$P2X_1$ mRNA is expressed in spinal cord (Collo et al., 1996) and in neonatal but not adult brain (Kidd et al., 1995). mRNA for the $P2X_2$ subunit is present in the brain, spinal cord and in sensory and sympathetic ganglia (Kidd et al., 1995; Collo et al., 1996). This mRNA is also present in the pituitary gland and adrenal medulla. Immunohistochemical studies performed with a $P2X_2$-specific antibody have confirmed the presence of the $P2X_2$ protein in these locations (Vulchanova et al., 1996). The $P2X_3$ subunit has a rather restricted localization pattern. Both the protein and its mRNA are present only on a subset of sensory neurons that are nociceptors, including those in trigeminal and nodose and dorsal root ganglia (Chen et al., 1995). $P2X_3$ immunoreactivity has been recently found in peripheral endings of a subset of nociceptive neurons present in the trigeminal ganglion projecting to the tooth pulp. Here, this receptor mediates nociception either as a homo-oligomer or in heteropolymerization with the $P2X_2$ subunit (Cook et al., 1997). In contrast, the $P2X_5$ receptor, whose mRNA also has a very restricted distribution, being present only in the cell bodies of proprioceptive afferens of the mesencephalic trigeminal nucleus (Collo et al., 1996), does not mediate nociception in sensory neurons. It may mediate mechano-sensation at the jaw muscle stretch receptor either as a homo-oligomer or in heteropolymerization with other subunits such as $P2X_2$ (Cook et al., 1997).

Transcripts for the $P2X_4$ and $P2X_6$ receptors are the most abundantly expressed P2X forms in the nervous system. They are found in the dorsal horn of the spinal cord and sensory ganglia. mRNAs for these P2X subunits are extensively present in large number of brain regions where they show marked overlapping distribution (Collo et al., 1996). $P2X_4$ mRNA was also found in salivary gland, where its native homo-oligomeric form displays the nondesensitizing, α,β-meATP and antagonist insensitive phenotype (Buell et al., 1996).

The $P2X_7$ receptor has a much wider distribution then has previously been suspected. mRNA for this subtype being not only present on macrophages and other related immune system cells, but in the heart, liver, pancreas, brain, spinal cord, skeletal muscle, lung, and spleen (Surprenant et al., 1996; Rassendren et al., 1997). In the brain the $P2X_7$ transcript appears to be present on ependyma and microglia, but not in neurons (North and Barnard, 1997). In the microglia, the $P2X_7$ mRNA is colocalized with other P2X subunits abundant in the brain (J. Simon, unpublished observation), raising the possibility for heteropolymerization. These cells are also thought to express subtypes of the P2Y receptor that may play an important role in channel modulation (discussed above). Microglial cells are the main immune effector cells of the brain and are known to release various cytokines and nitric oxid upon activation, in an analogous manner to macrophage activation (McGeer and McGeer, 1995) and are thought to play a role in various neurogenerative diseases. However, it is far from clear how the $P2X_7$ receptors, homo-oligomeric or heteropolimerized with other P2X subunits, play a role in physiological activities of microglia on ATP activation.

Genomic Organization and Alternative Splicing

Genomic organization of the rat $P2X_2$ (Brändle et al., 1997) and the human $P2X_7$ gene (Buell et al., 1997) have been determined to date. The $P2X_2$ gene contains 10 introns, ranging from 78 to 320 bp in the coding region, whereas the human $P2X_7$ has 12 introns in its coding region. The exon–intron boundaries show precise conservation for the first 10 introns. The large number of exon–intron boundaries present in the coding region suggest the existence of a number of subunit isoforms produced by alternative splicing. Interestingly, sequences encoding transmembrane regions are also interrupted by introns, raising the possibility of an existence of splice variants with slightly different TM domains. Recently three isoforms of the $P2X_2$ subunit were isolated from neonatal rat cerebellum (Simon et al., 1997a). The first variant, now termed $P2X_{2(b)}$, has a serine–proline rich region (207 bp) deleted from exon 11. This isoform was also obtained from rat cochlear tissue (Brändle et al., 1997). This short form of the $P2X_2$ subunit expresses in the nervous system at least as abundantly as the original $P2X_2$ subunit cloned by Brake et al. (1994), the latter now being termed $P2X_{2(a)}$ (Simon et al., 1997a). The $P2X_{2(b)}$ isoform is able to form homo-oligomeric functional channels, as was shown by its expression in *Xenopus* oocytes and in mammalian cells. The only difference in properties detected between this short and the $P2X_{2(a)}$ long form is an increased, intermediate rate of desensitization controlled by the serine-proline rich region at the C-terminal tail (*see above*). However, a dramatic change in the rate of desensitization occurs when two 34-residue segments containing the TM1 and TM2 region of the $P2X_1$ or $P2X_3$ subunits are exchanged with the corresponding sequences in the $P2X_{2(a)}$ sequence (Werner et al., 1996).

Alternative splicing also occurs in the TM1 region of the $P2X_2$ subunit, generating two additional isoforms (Simon et al., 1997a). In one isoform a 18 bp fragment is spliced out from exon 2 together with intron 1 ($p2x_{2(c)}$), using a cryptic splice site located in exon 2. In the second variant ($p2x_{2(d)}$) a 36-bp, in-frame insertion occurs at the same position. Although functional expression of these two isoforms has not yet been achieved (denoted by the lower case letters) mRNA for the $p2x_{2(c)}$ isoform is present in many structures in the nervous system, whereas transcript for the $p2x_{2(d)}$ variant has only been found in the nodose ganglion (Simon et al., 1997a). Two cDNAs encoding splice variants of the human $P2X_5$ subunit ($hp2x_{5(a)}$ and $hp2x_{5(b)}$) have also been deposited with Genbank (Table 1); however, no functional expression of these has yet been reported.

Chromosomal Localization of Human P2X Subunit Genes

Five of the seven known P2X subunits have been localized on human chromosomes. Genes for $P2X_1$ and $P2X_5$ have been mapped to the same locus (p13.3) on the human chromosome 17 (Buell et al., 1997). The human $P2X_4$ gene was locatized to chromosome 12, locus q24.32 (Garcia-Guzman et al., 1997), in very close proximity to the $P2X_7$ gene, mapped to the site of 12q24.2 (Buell et al., 1997).The human gene for $P2X_3$ was mapped to chromosome 11q12. These results indicate that some of the P2X subunit genes are clustered at the same locus and these are the subunits more likely to heteropolymerize, as is known for other transmitter-gated ion channels. Although, some of the loci where hP2X subunit genes are located are thought to contribute to several inherited disorders, none of these diseases could definitely be related as yet to any of the mapped hP2X genes.

Conclusion

P2X and P2Y Receptors in the Brain

Electrophysiological studies have accumulated evidence that ATP acts on both types of the P2 receptor in the brain and the P2X and P2Y receptors might coexpress in a number of regions throughout the brain. Some of these findings were recently reviewed by Illes et al. (1997). Both P2X and P2Y types of responses were observed in hippocampal neurons (Wierasko and Ehrlich, 1994; Balachandran and Bennett, 1996), hypothalamic neurons in culture (Chen et al., 1994), locus ceruleus in slices (Shen and North, 1993; Fröhlich et al., 1996) and rat medial vestibular nucleus (Chessell et al., 1997). *In situ* hybridization studies demonstrated that transcripts for the P2X receptor subtypes ($P2X_2$, $P2X_4$, and $P2X_6$) have a more distinct localization pattern than the $P2Y_1$ mRNA, the only P2Y subtype whose localization (Fig. 3) has been studied so far (Kidd et al., 1995; Collo et al., 1996; Webb et al., 1997). Whereas the P2X transcripts are mainly present on neurons (except the mRNA for the $P2X_7$ subunit) the $P2Y_1$ receptor has both neuronal and non-neuronal localizations (*see* Fig. 3). mRNAs for $P2X_2$, $P2X_4$, and $P2X_6$ together with the $P2Y_1$ transcript are all present in high density in cerebellar Purkinje cells, cerebellar cortex, in all hippocampal fields and dentate gyrus, as well as in the olfactory bulb (Kidd et al., 1995; Collo et al., 1996; Simon et al., 1997b; Webb et al., 1997). The $P2X_7$ subunit like the $P2Y_1$ receptor has a notable glial distribution. Furthermore, mRNAs both for P2X and P2Y subtypes have been detected by RT-PCR in a number of neuronal populations where electrophysiological and pharmacological measurements provided data characteristic of both P2Y and P2X receptors (J. Simon and T. E. Webb, unpublished observations). However, some of the data provided by electrophysiological measurements, or radioligand binding assays performed on brain membrane fractions or brain slices, cannot be explained by the localizations of the presently known P2X and P2Y receptors. For example, α,β-meATP sensitive responses have been recorded from neurons of the medial habenula (Edwards et al., 1992), medial vestibular nucleus (Chessel et al., 1997), and mesencephalic nucleus (Khakh et al., 1997). Autoradiography with [^{3}H] α,β-meATP on brain slices also located α,β-meATP sensitive binding sites (Bo and Burnstock, 1994; Balcar et al., 1995). However, none of the α,β-meATP-sensitive P2X receptor subtypes have been detected in the brain. Conversely, the two most abundant subtypes of the P2X receptor in the brain have little sensitivity to P2 antagonists, but no antagonist-insensitive responses have been detected from brain neurons so far. There may be a yet undetected P2X subunit or subunits in the brain with the above properties, or yet untested combinations of different P2X subunits may explain these discrepancies.

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